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Long-Term Hematopoietic Damage: Concepts, Approaches, and Results Relevant to the Study of Environmental Toxins

by Nydia G. Testa* and Thomas M. Dexter*

The hematopoietic tissue is one of the prime examples of hierarchical tissues, where mature cells with a limited life span are continually replaced as a result of proliferation and differentiation from stem and progenitor cells. In the bone marrow, these processes are controlled by growth factors and by cell to cell interactions, the latter being specially important for the regulation of the stem-cell population. In the study of long-term hematopoietic damage, we have to distinguish between deleterious effects of the environmental toxins on the stem and progenitor cells, and on the stromal bone marrow cells which are part of the regulatory hematopoietic microenvironment. In some experimental situations, the function of the tissue may be subnormal, not because of stem cell damage (which may also be present), but because of damage to regulatory environmental populations. Because of the high degree of organization of the hematopoietic tissue (not immediately obvious from histological sections), the heterogeneity of the stromal cell populations, and their different capacities to regenerate after cytotoxic insult, the stromal damage is likely to be heterogeneous and would tend to be expressed functionally at later times than acute hematopoietic injury. While microenvironmental damage may be of importance in the induction of hematopoietic failure, the primary target in leukemogenesis is likely to be the stem cell. However, experimental data support the concept that regulatory microenvironmental influences may hinder or favor the development of leukemia.

Introduction

The continuous physiological replacement of vast numbers of mature hematopoietic cells is the end result of ordered processes of cell proliferation, differentiation, and maturation that start at the level of hematopoietic stem cells. Stem cells generate progenitor cell populations with more limited proliferation capacity, which are able to differentiate only along one or two hematopoietic lineages. Progenitor cells will, in turn, give rise to the more mature cells that are recognizable morphologically and that comprise about 99% of the hematopoietic cells in the bone marrow.

The hematopoietic tissue has the capacity to respond quickly and effectively to increased demand for mature cells, for example, to replace blood loss or to deal with infection and can maintain this response for prolonged periods of time. The finely tuned mechanisms involved in the regulation of progenitor cells are becoming relatively understood, but less is known about the regulation of stem cells, either their self-reproduction (a crucial factor in maintaining hematopoietic function in the long term) or their commitment to differentiation. Here, we shall analyze the relative contribution of damage to stem and progenitor cell populations and to the regulatory microenvironment within which hematopoiesis takes place to the long-term function of the tissue.

Structure of the Hematopoietic Tissue

The first clonal assay in experimental hematology was based on the observation that after the injection of bone marrow cells into lethally irradiated mice, some of the injected cells (CFU-S) lodge in the spleen and, by clonal proliferation, give rise to visible colonies that contain not only cells of several hematopoietic and lymphoid lineages, but also cells able to generate further spleen colonies upon retransplantation (*1-3*).

There is, however, great heterogeneity in the measured potential of the spleen colony-forming cells: some of the CFU-S are not multipotential or able to self-reproduce, and may only be able to give rise to committed progenitors and to cells of only one hematopoietic lineage (*4,5*). However, at least some spleen colony-forming cells can be equated to stem cells, and the CFU-S assay remains widely used as an indirect, but reliable, estimate of the stem cell capacity of the tissue in several

*Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Withington, Manchester M20 9BX, UK.

Address reprint requests to N. G. Testa, Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Withington, Manchester M20 9BX, UK.

experimental approaches. The existence of stem cells has been elegantly demonstrated by showing that the hematopoietic and lymphoid systems of a potentially lethally irradiated mouse can be repopulated by the progeny of a single cell, which can be detected by the presence of either a chromosome marker or an inserted retroviral marker (*6,7*). A multipotential cell, which overlaps with and is able to generate CFU-S, is the murine mixed colony-forming cell (Mix-CFC), detected by an *in vitro* clonal assay (*8*). A similar cell can be detected in humans (*9*). Committed progenitor cells (CFCs) that give rise to the different hematopoietic lineages are also studied using *in vitro* clonal assays (*10*) where they are induced to proliferate in response to hematopoietic growth factors (colony-stimulating factors, CSFs). The hierarchical organization of these cell populations is shown in Figure 1.

Regulation of Hematopoiesis

The hematopoietic growth factors, commonly referred to as colony-stimulating factors (CSFs) (Table 1) (*11–14*) were first detected by their capacity to induce proliferation and maturation of colony forming cells *in vitro*. Some of them appear to be largely lineage restricted, with M (macrophage)-CSF acting on cells whose proliferation will result only in the production of macrophages, and G (granulocyte)-CSF, inducing production of neutrophilic granulocytes. In contrast, interleukin 3 (IL-3) will promote not only the development of the committed progenitor cells, but will also stimulate the development of Mix-CFC. Similarly, GM-CSF will not only stimulate the production of neutrophilic granulocytes and macrophages, but will also stimulate progenitor cells to produce eosinophils and megakaryocytes. These two factors will also induce red cell development from primitive progenitor cells in the presence of erythropoietin. Other growth factors isolated more recently may also have a wider range of action than initially thought (Table 1). Other molecules, collectively described as biological response-modifiers, can influence hematopoiesis directly or indirectly, although by themselves they do not act as growth factors (i.e., they cannot induce colony formation by progenitor cells). They include prostaglandins, interferons, tumor necrosis factor, and transforming growth factors. Among them there are also some interleukins that have effects on myeloid and lymphoid cells, but their mode and range of action have yet to be established. A comprehensive list of effects is found in O'Garra et al. (*15*). The most relevant here is interleukin 1 (IL-1) which, per se, does not stimulate colony formation. In this context, it was first described as a potentiation factor called hematopoietin 1, which induces primitive progenitor cells into a state in which they are able to respond to M-CSF stimulation, with production of macrophages (*14*). Whether this is the only, or even the main effect of IL-1 on hematopoiesis remains to be determined.

The role of growth factors in hematopoiesis was elucidated by devising experimental *in vitro* systems where the action of purified factors on purified target cells could be investigated. However, the bone marrow is a complex heterogeneous tissue with a well-defined anatomical structure. Although bone marrow sections examined microscopically show an apparently random collection of cells, determinations of the incidence of colony-forming cells and nonhematopoietic stromal cells show a nonrandom, nonhomogeneous distribution of different cell populations within the marrow space in mice (*12*). The data for humans is more limited (*16*). Also, cell populations that produce regulatory factors are more concentrated in certain areas. This presumably leads to different regulatory influences being exerted in local domains. By using long-term bone marrow cultures, it has been established that complex cell interactions influence the growth and development of stem cells. In these cultures stromal cells (fibroblastic-reticular cells, adipocytes, endothelial cells)

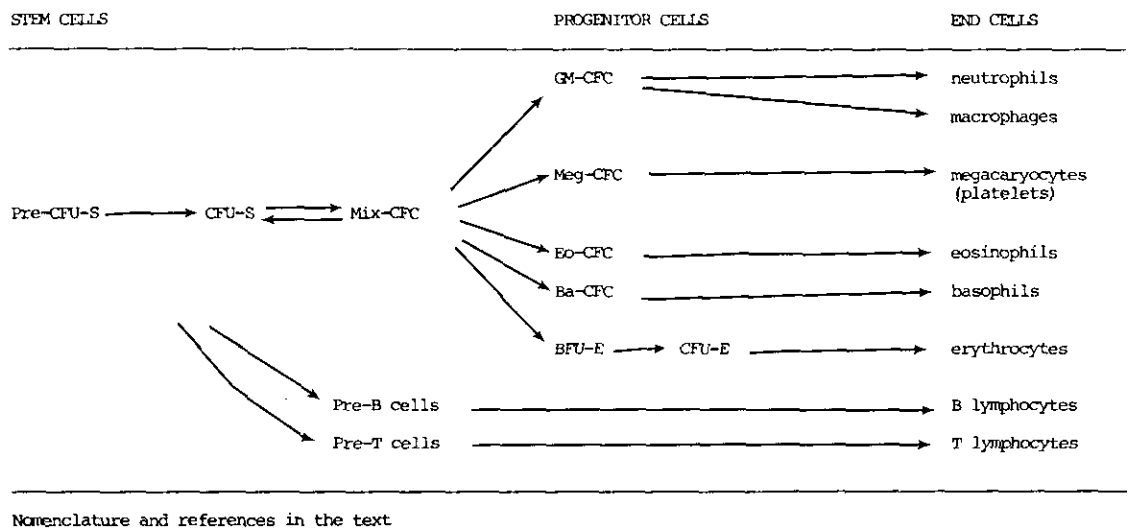

FIGURE 1. Cell hierarchies in hematopoiesis.

Table 1. Growth factors and target *in vitro* colony-forming cells (CFC).

Growth factor	Mix	Granulocyte-macrophage	Erythroid		Megakaryocytic	Eosinophilic
			Early CFC	Late CFC		
IL-1	+	?	?	?	?	?
IL-3	+	+	+	+	+	+
IL-4	+	+	+	+	+	?
IL-5	–	–	–	–	–	–
IL-6	?	?	?	?	?	?
GM-CSF	–/+	+	–/+	–	+	+
M-CSF	–	+	–	–	–	–
G-CSF	–	+	–	–	–	–
Megacaryocytes-CSF	–	–	–	–	+	–
Erythropoietin	–	–	–	+	+	–

[a](+) Stimulation; (–) no effect; (?) not known.

form a complex multilayer attached to the surface of culture flasks, forming a microenvironment within which active hematopoiesis may take place for several months (*14*). Hematopoietic growth factors, such as M-CSF and GM-CSF may be produced within the adherent layer, but they are not released at significant levels into the growth medium. IL-3, however, has not been detected in such cultures (*14*). This culture system, unlike the clonal assays for progenitor cells, allows sustained renewal and differentiation of stem cells. These events are likely to be crucially influenced by the capacity of stem cells to bind to stromal cells, a step critical before stromal cell-bound regulatory factors may influence them. This step may also be needed before growth factors bound to extracellular matrix molecules (themselves cell-bound?) (*14,17*) may interact with the stem cells.

It follows that events that may damage the regulatory milieu of stem cells can be studied using long-term cultures. As will be seen from the following discussions, instances where defects in the regulatory stroma may be the causative factor in subnormal hematopoiesis have been documented.

Long-Term Hematopoietic Injury

More is known about long-term damage after cytotoxic agents (drugs and radiation) used as therapy for malignant disease than about the effect of environmental toxins. Clearly, one important reason for this is that studies relevant to the therapy of malignancy usually deal with maximum tolerated doses where doses are known and where clear effects are observed, whereas a frequent problem in environmental studies is to decide where a dose threshold lies (and, indeed, whether a threshold exists). Also, determining dose-effect relationships by measuring biological effects that are close to the limits of methodological resolution is a problem after low doses of toxins.

Fundamental differences, for example, in drug metabolism between species of mammals used in experimental work and between these mammals and humans complicate the assessment of damage. However, epidemiology can establish convincing associations between hematopoietic damage and environmental toxins, even if the degree, type, and extension of such damage may not be easily determined. Here, we shall use examples of experimental studies using drugs or radiation to illustrate types of hematopoietic damage and the adaptive response of the tissue, which may be relevant to the study of environmental toxins.

Stem Cell Damage Versus Microenvironmental Damage

Direct damage to stem cells is to be expected after exposure to cytotoxic agents. Just depletion of stem cell numbers, however, is not a likely cause of hematopoietic failure leading to hypoplasia or aplastic anemia. It is known that the CFU-S population is not reduced after recovery from severe and repeated proliferation stress induced by depletion *in situ*. Repeated administration of triethilenmelamine (*18*) or hydroxyurea (*19*) at doses that each produced a depopulation of the order of 99% of CFU-S, was followed by recovery of CFU-S to normal numbers (*18*). Furthermore, the quality of these cells, i.e., their self-reproduction capability, was maintained (*19*). After depletion by other treatments, however, CFU-S numbers never recover to control levels (*20*). After depletion by busulphan, a phase of exponential growth follows, but CFU-S reach only about half the numbers in the untreated controls. Suboptimal plateau numbers of CFU-S are also found after treatment with other agents (*20*). There are several interesting features in these new steady states that may follow drug treatment:

a) The level to which the CFU-S recover is not related to the acute CFU-S kill. Over a range of doses up to the maximum tolerated dose, following single or repeated administration of busulphan, cyclophosphamide or *bis*-cloroethyl nitrosourea (BCNU), the CFU-S recover to a level characteristic of the drug but are not influenced by the dose used (*21-23*). The level is stable for the rest of the life-span of the mice. After radiation, however, the level of the suboptimal plateau is related to dose.

b) The GM-CFC population plateaus at a higher level than the CFU-S (*22,23*).

c) In spite of *a* and *b* above, the mature cell output (i.e.,

peripheral blood counts) may be normal because of several compensatory mechanisms (e.g., increased amplification from the progenitor cell compartment) that are called into action (*16*). However, when the system is stressed by transplantation, fewer cells are produced than in controls (*22*). Also, in some cases, notably after busulphan, a late hypoplasia develops (*24*) in the treated mice. A similar picture may develop after repeated radiation treatment (*25*). It is not clear whether this syndrome should be considered as a preleukemic state.

d) The suboptimal plateau in CFU-S, seen after treatment with some cytoreductive regimes, is not determined by a reduced self-reproduction or proliferation capacity of these cells (although those effects may be present). When transplanted into appropriate recipients the CFU-S are capable of fast exponential growth and often complete regeneration of the hematopoietic system.

Thus, it is clear that the subnormal hematopoiesis that may be seen in treated mice may not be (or at least not primarily) a function of stem cell damage. In many cases the subnormal plateau in CFU-S numbers could well be a limitation imposed on them by a damaged regulatory microenvironment. There is other evidence of microenvironmental damage as the main course of disturbed hematopoiesis after injury: Following treatment with cyclophosphamide, the CFU-S with the highest self-reproduction capacity are spared selectively. From this, one would expect a good reconstitution of the CFU-S compartment. However, as stated earlier, subnormal numbers are observed and in a minority of mice there is serious hematopoietic damage manifested as hypoplasia or myelodysplasia (*21*). That this may be associated with damage to the microenvironment is indicated by the finding that a stromal cell population of reticular fibroblastoid cells is also subnormal (*21*). There is also evidence that microenvironmental abnormalities may cause aplastic anemia in humans (*26–29*).

In the context of microenvironmental damage by toxins, it is important to consider not only what the basic lesions are, but also (and perhaps more importantly) which are the limiting factors that govern cell production when hypoplastic syndromes are considered. It would appear from the experimental data reviewed previously that even in the presence of stem cell damage, the crucial factor in determining the level of hematopoiesis is the damage to the regulatory microenvironment. When induction of leukemia is considered, damage to stem cells is the most likely primary consideration. Microenvironmental influences, however, are also likely to play a role in leukemogenesis. It is known that myeloid leukemic cells respond to hematopoietic growth factors, and indeed require them for growth *in vitro* (*30*). Furthermore, the regulatory stroma in long-term cultures favors the reappearance of normal hematopoiesis when bone marrow from patients with acute myeloblastic leukemia is cultured. The leukemic clonal cells may become undetectable, and normal, presumably polyclonal, cells reappear and become predominant (*31,32*).

Assays for the Study of Microenvironmental Damage

Several assays to study the hematopoietic microenvironment are listed in Table 2. The first described is a clonal assay that detects a cell population (CFU-F) able to give rise to colonies of fibroblastoid-reticular cells in culture (*33*). Persistent low numbers of CFU-F are found after acute recovery from treatment with busulphan (following a pattern strikingly similar to that shown by CFU-S) and also after cyclophosphamide or radiation (*21,23,25*).

Implantation of femoral bone SC in mice results in colonization of the bone cavity by hematopoietic cells of the host, supported by the donor stroma which develops *in situ*. Low numbers of CFU-S are supported by the stroma of busulphan-treated mice (*35*). Further evidence of microenvironmental damage after busulphan is observed after implanting bone marrow tissue under the kidney capsula of mice, a technique originally described by Friedenstein et al. (*36*). In this assay, a donor-derived microenvironment develops at the site of implantation, which is able to support host-derived hematopoiesis. The environment provided by marrow from busulphan-

Table 2. Methods for the study of the microenvironment.[a]

Method	Species	Parameters studied
CFU-F[b] assay	Guinea pig, mouse, human, rabbit, rat, cat	Colony forming incidence, cell type, production of collagen, production of CSF
Long-term bone marrow cultures	Mouse, human, tree-shrew, cat	Hematopoiesis, stem and progenitor cell production, production of hematopoietic growth factors, study of the extracellular matrix, normal and leukemic cell differentiation, types and function of stromal cells
Subcutaneous implants	Mouse (femoral shaft)[c] Rabbit (bone marrow, demineralized bone)[c]	Hematopoietic foci, origin of stromal and hematopoietic cells, bone formation, fat accumulation, production of CSF, production of collagen
Implants under the kidney capsule	Guinea pig, rabbit, mouse (bone marrow, cultured fibroblasts)[c]	Bone formation, hematopoietic foci, origin of stromal and hematopoietic cells

[a]Methodology reviewed in Testa and Gale (*42*).
[b]CFU-F, colony-forming unit, fibroblastic.
[c]Tissues in parentheses are implants.

treated mice is able to support hematopoiesis only at about 50% of the control level (*23*).

In long-term bone marrow cultures active hematopoiesis depends, as described above, on the formation of a complex adherent layer of stromal cells (*14*). The level of hematopoiesis depends on the functional integrity of the stroma. When normal bone marrow cells are inoculated onto preformed adherent stromal cell layers derived from marrow of busulphan-treated mice, the microenvironment they provide is only able to support about 20% of the level of hematopoiesis seen in cultures where the stroma was provided by marrow of untreated mice (*37*). In the converse experiment, where marrow from treated mice was inoculated on normal adherent layers, the level of hematopoiesis was about 80% of controls (normal on normal). This again indicates that the limiting factor here is the microenvironmental, not the stem cell damage. A similar situation is found after chemotherapeutic treatment for lymphoblastic leukemia (*38*). The considerable reserve capacity of stem cells to respond to intensive proliferative stress, calculated to be able to support hematopoiesis for at least five life-spans in mice (*39*), may still be considerable even in a damaged stem cell population that is consequently able to comply with the demands for mature cell production. However, drugs and chemicals, unlike radiation, may have selective effects on certain subpopulations of the stem cell hierarchy. Those agents that damage the earliest cells (i.e., those cells with the highest self-reproduction and repopulation capacity) are more likely to induce stem cell failure than those that hit selectively more mature populations (*20*).

The capacity of stromal cells to recover after cytotoxic injury is likely to be considerably less than that shown by stem cells. At least some types of stromal cells have a slow turnover (*40*) and may also have a long life-span. Considerable differences in those parameters in the different types of stromal cells is also to be expected. Also, injury to them may take a long time to be expressed functionally. For example, the microenvironmental cells in long-term bone marrow cultures can be sterilized by large doses of radiation, but they remain able to support hematopoiesis for 4 to 6 weeks (*41*). It is only when the sterilized cells attempt to divide that they will be eliminated. In this context it is of interest to note that transient normalization of CFU-S may be observed at about 100 days after busulphan treatment. Normal values, however, are not maintained (*23*). Another important concept is that not only the integrity of several cell populations (with different life-spans and different proliferative potential to recover after injury) may be required for adequate regulatory function, but also the correct cell associations may have to take place and the correct ratios of different cell types maintained (*42*).

Studies of the hematopoietic system after exposure to benzene have so far concentrated on the acute injury. These studies have demonstrated that CFU-S and CFCs are sensitive targets for this agent. However, microenvironmental damage has also been reported. These data are reviewed elsewhere in this volume. Long-term studies of hematopoietic and stromal cell populations are of obvious importance in trying to understand the induction of aplasia and leukemia after exposure to benzene.

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